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Robert E. Hanson

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:
Gabrilovich *et al.*

Serial No.: 09/526,320

Filed: March 15, 2000

For: DENDRITIC CELLS TRANSDUCED WITH A WILD-TYPE SELF GENE ELICIT POTENT ANTITUMOR IMMUNE RESPONSES

Group Art Unit: 1632

Examiner: Wehbe, A.

Atty. Dkt. No.: INRP:074US

REPLY BRIEF

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REPLY BRIEF

MS Appeal Brief - Patents
Commissioner for Patents
P. O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

Appellants hereby submit an original and three copies of this Reply Brief in response to the Examiner's Answer, dated December 19, 2003. It is believed that no additional fees are due; however should any other fees be due the Commissioner is authorized to withdraw the appropriate fees from Fulbright & Jaworski Deposit Account No. 50-1212/INRP:074US.

Please date stamp and return the enclosed postcard to evidence receipt of this document.

I. STATEMENT OF INTEREST

The real parties in interest are the assignees, Introgen Therapeutics, Inc., Austin, TX and Vanderbilt University, Nashville, TN.

II. RELATED APPEALS AND INTERFERENCES

There are no related appeals or interferences.

III. STATUS OF THE CLAIMS

Claims 1-11, 15-22, 24, 26-31, 33-37 and 61-135 were pending at the time of the final Office Action. Claims 5-10 and 61-135 were canceled in an Amendment under 37 C.F.R. §1.116 filed concurrently with the Appeal Brief on May 14, 2003. The Examiner's Answer indicates that the Amendment has been entered. Therefore, claims 1-4, 11, 15-22, 24, 26-31 and 33-37 were pending and the subject of the appeal at the time of the Examiner's Answer.

Appellants hereby withdraw the appeal of the rejection of claim 1 to focus the appeal on the species of "tumor suppressor genes" elected for examination on the merits in the Response to Restriction Requirement dated 8/22/01. Claims 2-4, 11, 15-22, 24, 26-31 and 33-37 are therefore currently pending and the subject of the Appeal. A copy of the appealed claims is attached as **Appendix 1** and a copy of the pending claims is attached as **Appendix 2**.

IV. STATUS OF THE AMENDMENTS

An Amendment Under 37 C.F.R. §1.116 canceling non-elected claims 5-10 and 61-135 was submitted with the Appeal Brief filed on May 14, 2003. The Amendment has been entered by the Examiner.

V. SUMMARY OF THE INVENTION

The present invention addresses the need for an improved method for treating a human subject having or suspected of having cancer or pre-cancerous disease comprising the steps of (i) identifying a subject having or suspected of having cancer or pre-cancerous disease characterized by alteration or increased expression of a self gene product in at least some of the cancer or pre-cancerous cells in said subject; and (ii) intradermally administering to said subject an expression construct in an adenovirus particle comprising a self gene under the control of a promoter operable in eukaryotic dendritic cells, wherein the dendritic cells are infected by said construct, whereby said self gene product is expressed by dendritic cells and presented to immune effector cells, thereby stimulating an anti-self gene product response. Specification at least on page 5, lines 26 to 27; page 6, lines 14 to 21 and pages 28 to 31.

The self-gene product can be an oncogene. Specification at least on page 6, line 23 and table 1.

In certain embodiments, the oncogene is selected from the group consisting of tumor suppressors, tumor associated genes, growth factors, growth-factor receptors, signal transducers, hormones, cell cycle regulators, nuclear factors, transcription factors and apoptic factors. Specification at least on page 6, lines 23 to 26 and table 1.

In another aspect, the tumor suppressor is selected from the group consisting of Rb, p53, p16, p19, p21, p73, DCC, APC, NF-1, NF-2, PTEN, FHIT, C-CAM, E-cadherin, MEN-I, MEN-II, ZAC1, VHL, FCC, MCC , PMS1, PMS2, MLH-1, MSH-2, DPC4, BRCA1, BRCA2 and WT-1. Further, the tumor suppressor product can be p53. Specification at least on page 6, lines 26 to 30 and 14 to 18.

In certain embodiments, the adenovirus particle can be replication-defective. The replication defect can be a deletion in the E1 region of the virus. In particular embodiments, the deletion maps to the E1B region of the virus. The deletion can encompasses the entire E1B or E1 region of the virus. Specification at least on page 7, lines 19 to 23 and pages 31 to 35.

In certain embodiments, the promoter is selected from the group consisting of CMV IE, human or murine dectin-1, human or murine dectin-2, human CD11c, mammalian F4/80 and human or murine MHC class II. In particular embodiments, the promoter is CMV IE. In another aspect, the expression vector further comprises a polyadenylation signal. Specification at least on page 7, lines 25 to 28 and pages 44 to 48.

In certain embodiments, the cancer is selected from the group consisting of lung, head, neck, breast, pancreatic, prostate, renal, bone, testicular, cervical, gastrointestinal, lymphoma, brain, colon, skin and bladder. Specification at least on page 8, lines 1 to 7 and page 51.

In particular embodiments, the expression construct is administered via injection. Specification at least on page 8, lines 17 to 18; page 52 and page 64.

In another aspect, the expression construct is administered via injection comprising multiple injections. Specification at least on page 8, lines 17 to 18 and page 52.

Embodiments of the invention include a method wherein the injection is performed local, regional, or distal to a cancer, a pre-cancer or a tumor site. Specification at least on page 8, lines 18 to 22 and page 52.

Embodiments of the invention include a method wherein intradermal administration is via continuous infusion. Specification at least on page 8, lines 22 to 23 and page 65.

In certain embodiments, the immune effector cells are CTLs. Specification at least on page 8, line 15 and page 26.

In another aspect, a method further comprises administering to said subject at least a first cytokine. The method may further comprising administering to the subject a second cytokine, different from the first cytokine. The cytokine may be selected from the group consisting of GM-CSF, IL-4, C-KIT, Steel factor, TGF-β, TNF-α and FLT3 ligand. In certain embodiments, the cytokine is administered as a gene encoded by the expression construct. Specification at least on page 8, lines 9 to 15.

VI. ISSUES ON APPEAL

Do claims 2-4, 11, 15-22, 24, 26-31 and 33-37 satisfy the enablement requirement of 35 U.S.C. 112, first paragraph?

VII. GROUPING OF THE CLAIMS

For the purposes of this Appeal, the claims stand or fall together.

VIII. SUMMARY OF THE REPLY

The Examiner's Answer fails to provide any objective basis to doubt the enablement of the appealed claims, particularly when taken with the numerous lines of evidence provided by Appellants in support of enablement. The Examiner relies on the unsupported contention that the working examples do not support the claims because *in vivo* transduction of dendritic cells is not enabled. Appellants have further affirmatively demonstrated that intradermal delivery of adenoviral vectors yields a potent immune response. Coupled with the working examples demonstrating tumor protection by *in vivo* delivery of dendritic cells transduced *ex vivo* with Ad-p53, the rejection is completely insufficient on its face.

The only "support" provided for the rejection are numerous sound bites culled from the prior art alleged to show shortcomings of "gene therapy." However, the Examiner has failed to show that these references have any relevance to the objective enablement of the appealed claims. In view of the insufficiency of the scientific reason for doubting the enablement of the appealed claims, and the objective evidence submitted in support of enablement, reversal of the rejection is respectfully requested.

IX. STATEMENT OF COMPLIANCE WITH 37 C.F.R.§1.195

Appellants attached Exhibits K and L were not presented earlier because they were only called to the attention of the undersigned after receipt of the Examiner's Answer. Submission of the Exhibits is therefore believed to be in compliance with 37 C.F.R. §1.195. In the event that the Examiner disagrees, Appellants submit that the previously-presented Exhibits more than adequately support the appeal and thus request consideration of the appeal based on the original Exhibits.

X. REPLY

A. The Examiner's Rejection is Premised on the Alleged Inability to Transduce Dendritic Cells With *In Vivo* Adenoviral Delivery

The Examiner's rejection is premised on the incorrect conclusion that Appellants have not demonstrated enablement for *in vivo* delivery of expression constructs to dendritic cells by intradermal administration of adenoviral particles. For example, the Examiner acknowledges that the working examples illustrate inhibition of growth of a p53 positive tumor line by administration of dendritic cells that were transduced *ex vivo* with adenoviral particles (Ad-p53), but asserts that there is no nexus with the claims because of the alleged inability to transduce

dendritic cells *in vivo*. Examiner's Answer at p 8. The Examiner contends in particular that *in vitro/ex vivo* transduction occurs "under artificial cell-culture conditions which allow for direct contact of the dendritic cells with optimized levels of the virus." It is further asserted that, in contrast, *in vivo* transduction is affected by many variables, such as rate of vector clearance from the injection site, tropism of the vector for the target cell, the rate of cell transduction under physiological conditions and the presence of any pre-existing immune responses to the virus itself. Based on these alleged "substantial differences" the Examiner concludes that "a nexus between the *ex vivo* working examples and the *in vivo* methods claimed cannot be found" and therefore the working examples do not support enablement of the claims. Examiner's Answer at p. 9, 1st ¶.

The problem with the Examiner's argument and reason why the rejection must be reversed is that Appellants have in fact demonstrated enablement for *in vivo* transduction of dendritic cells by intradermal administration of adenoviral particles. As explained in detail in Section B below, Appellants have provided numerous lines of evidence showing the effectiveness of *in vivo* transduction of dendritic cells by intradermal injection of adenoviral particles. Having demonstrated enablement for *in vivo* transduction of dendritic cells, the remainder of the Examiner's rejection falls apart based on the working examples in the specification showing that delivery of dendritic cells transduced with Ad-p53 yields a significant anti-tumor immune response. Specification at pp. 72-73.

B. Appellants Demonstrate Enablement for *In Vivo* Transduction of Dendritic Cells

As noted in Appellants Brief, peer-reviewed scientific publications clearly demonstrate that intradermal administration of adenoviral vectors yields a potent immune response. For

example, Gilbert *et al.* (*Vaccine* 15;20(7-8):1039-45, 2002, **(Appeal Brief Exhibit H)** found that recombinant replication-defective adenovirus expressing the CS gene from *Plasmodium berghei* (Ad-PbCS) induced a strong CD8(+) T cell response after intradermal or muscular injection. Gilbert *et al.* at §3.1 and fig. 1 on p. 1041. Fig. 1 of Gilbert *et al.* shows that intradermal administration of recombinant adenovirus yielded a greater peptide-specific immune response than the other routes of administration used, including subcutaneous, intransal and intramuscular. Fig. 1 at p. 1041.

The Examiner's Answer disregards this study based on the assertion that the antigen that was used is a strong parasitic antigen "unlike the self gene products recited in the instant methods." Examiner Answer at p.19. The Examiner also asserts that low or no level of protection to infection with the parasite *P. berghei* was obtained. However, the former comment goes to the ultimate immune response that would be expected to be obtained, not the transduction of DCs regardless of the antigen expressed. A response would not be expected in the first place without transduction of DCs. With regard to protection from parasite challenge, this is irrelevant to the claims which concern cancer and not parasites. Lack of protection from parasites does not equate with lack of protection from tumors. The Examiner's position falls apart in this instance because Appellants have already shown that DCs transduced *ex vivo* with p53 induce a potent anti-tumor immune response. Further, the reference shows that intradermal administration of Ad followed by a modified vaccinia virus (MVA) yielded a complete protection, nearly three times the rate of successive MVA injections, thus intradermal Ad injections clearly yielded an immune response.

The Examiner disregards evidence in Kaiserlain *et al.* (1999) **(Appeal Brief Exhibit C)** supporting enablement because the gene delivery involved administration by tape-stripping the

corneal layer of the skin followed by application of the adenovirus by "occlusive technique." The Examiner asserted that this method was not included in the specification and therefore did not support enablement. However, at page 58 of the specification in the section entitled "Additional Modes of Delivery" it is stated that methods of self gene delivery contemplated include ophthalmic formulations, citing Bourlais *et al.*, 1998 ("Opthalmic drug delivery systems-recent advances." *Prog. Retin. Eye Res.*, 17:33-58). This paper is incorporated by reference in the "References" section of the application. It is therefore submitted that delivery of adenovirus to the corneal layer of the skin with techniques such as that used by Kaiserlain *et al.* is fully enabled by the specification. In any event, the significance of the reference is the efficacy of dendritic cell priming with Ad vectors delivered to DC *in vivo*, which is illustrated by the paper. The Examiner has therefore failed to explain away this additional line of evidence.

The study by Bonnotte *et al.*, (*Cancer Research* 63:2145-2149 (2003); attached as **Exhibit K**) demonstrates that dermal sites have a high density of dendritic cells that "facilitates the capture of tumor antigens and that local inflammation induces maturation of the DCs and their migration into draining lymph nodes." This study involved introduction of tumor cells cultured *ex vivo*, but illustrates the effectiveness of intradermal injection for presentation of tumor antigens. In fact, Bonnotte *et al.* note that when injected intradermally, "normally nonimmunogenic tumor cells can induce an immune response and be rejected." p. 2147, 2nd col., 1st full ¶. The authors conclude by stating that "the richness of DC in skin should make i.d. site a preferable site for injection of modified tumor cells in experimental and clinical trials using tumor vaccines." P. 2149. The study therefore illustrates that intradermal injection provides access to a rich source of DCs that are highly amenable to uptake of tumor antigen.

Finally, Hirschowitz *et al.*, (*Gene Therapy* (1998) 5:975-983; attached as **Exhibit L**) showed that intradermal adenoviral delivery of the gp75 melanoma protein resulted in a significant anti-tumor response against melanoma. Specifically, a single intradermal administration of Ad-gp75 to mice 7 days before tumor cell challenge provided "marked protection" against tumor cell challenge based on a decrease in the number of metastases to 29% +/-4% relative to the control. This study specifically demonstrates that intradermal injection of adenoviral delivered self antigens may be used to obtain a potent immune response against tumor cells.

While, unlike the appealed claims, the Hirschowitz *et al.* study did not involve a tumor suppressor gene and one of ordinary skill in the art could not have predicted at the time that tumor suppressor genes would yield an anti-tumor response, Appellants' *ex vivo* studies bridge this gap. Specifically, Appellants' demonstration that 17 of 20 (85%) mice immunized with Ad-p53 DC were completely protected against D459 tumor cells and 8 of 11 mice (72.7%) were protected against MethA sarcoma demonstrates the ability to obtain a potent anti-tumor activity by Ad delivery of tumor suppressor genes to DC. The evidence presented by Appellants demonstrates that such tumor suppressor genes can be delivered *in vivo* and *ex vivo* and thus the Examiner's rejection is without basis.

C. The Examiner Has Failed to Show That the Working Examples Are Not Representative of the Appealed Claims

The Examiner attempts to support the rejection by citing the alleged heterogeneity of tumor suppressor genes. Examiner's Answer at p. 21. It is stated in particular that tumor cells employ mechanisms to evade tumor challenge and that obtaining an immune response to self antigens is unpredictable because of natural tolerance to self-antigens.

With regard to the alleged inability to obtain an immune response to self antigens, Appellants once again note that the working examples illustrate an immune response to the ***self tumor suppressor gene product p53***. No basis has been provided to conclude why an immune system would respond to this self gene product, but not other self tumor suppressor genes. Further, the comments of the Examiner go to the innate immune systems response to self-antigens, not ***stimulated*** immune responses.

Hurpin *et al.*, ((1998) *Vaccine*, Vol. 16, No. 2/3, 208-215) demonstrate that "***more than 50% of tumors overexpress p53***." (emphasis added) (**Appeal Brief Exhibit G**). This strongly contradicts the heterogeneity asserted by the Examiner. Appellants' working examples therefore directly apply to more than half of tumors based on the p53 studies alone. The Examiner nonetheless merely alleges that enablement is lacking because there are many other tumor suppressors. However, other tumor suppressor genes were well known in the art and are described in the specification (*see, e.g.*, specification at pp. 28-31). Any alleged heterogeneity in these tumor suppressors is irrelevant in view of the guidance given and the lack of any basis to conclude that they would behave differently than p53. The Examiner's comments regarding self gene products are also strongly contradicted by the illustration in Hirschowitz *et al.* (**Exhibit L**) of a potent anti-tumor immune response following a single intradermal administration of the self-antigen gp75 in mice. Although admittedly not a tumor suppressor gene, the working examples bridge this difference.

The Examiner has done nothing more than doubt enablement of the claims without providing a reasonable objective evidentiary basis for doing so. In view of Appellants' affirmative evidence supporting enablement, it is respectfully submitted that the rejection must be reversed.

D. The References Cited by the Examiner Fail to Support the Enablement Rejection

Throughout the Answer the Examiner cites short sound bites culled from the prior art expressing doubts regarding "gene therapy" techniques without specifying the particular techniques doubted. For example, Verma *et al.* (**Appeal Brief Exhibit D**) is said to report that "[t]he Achilles heel of gene therapy is gene delivery" and that "most of the approaches suffer from poor efficiency of delivery and transient expression." Marshall (**Appeal Brief Exhibit E**) and Orkin *et al.* (**Appeal Brief Exhibit F**), are cited as supporting the unpredictability of gene therapy generally and that some problems remain, mainly with regard to therapies that require replacement of defective genes, long term expression of genes and/or the transfection of large number of cells *in vivo*. Hurpin *et al.* (**Appeal Brief Exhibit G**) is cited as demonstrating that the route of administration has substantial effects on the ability to generate an immune response.

The problem with the references cited by the Examiner is that they have not been shown to be relevant to the ***claimed invention***. Appellants do not claim "gene therapy" generally but rather claim a specific procedure for treating a subject with cancer. The fact that previous different therapeutic methods may or may not have had subjective clinical efficacy is no reflection on the objective enablement of the appealed claims. For example, Verma *et al.*, Marshall and Orkin *et al.* do not address priming of ***dendritic cells*** using ***adenoviral vectors*** for producing an immune response against cancer. Hurpin *et al.* show a difference in ability to generate a CTL response using intradermal administration of a particular ***poxvirus*** construct and plasmid vectors, but not intradermal administration of ***adenoviral particles***.

Indeed, the Examiner has failed to show that a single reference is relevant to intradermal injection of adenoviral particles. In contrast, Appellants *ex vivo* working examples demonstrate

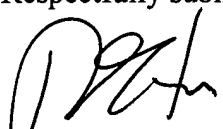
the propensity of adenovirus for dendritic cells. The working examples also illustrate that dendritic cells transduced *ex vivo* with adenovirus-delivered p53 tumor suppressor result in a potent anti-tumor immune response. Any alleged lack of nexus between the working examples and the claims is eliminated by the references submitted showing the effectiveness of *in vivo* gene delivery by intradermal injection of adenoviral particles.

In view of the working examples in the specification, the insufficiency of the scientific basis for doubting the enablement of the appealed claims, and the objective evidence submitted in support of enablement, Appellants have more than adequately demonstrated the enablement of the appealed claims. Reversal of the rejection is thus respectfully requested.

XI. CONCLUSION

In light of the foregoing, Appellants respectfully submit that the claims on appeal should not be rejected under 35 U.S.C. § 112, first paragraph as not being enabled. Reconsideration and withdrawal of the rejection is requested.

Respectfully submitted,



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Date: February 19, 2004

APPENDIX 1: APPEALED CLAIMS

2. The method of claim 1, wherein said self-gene product is an oncogene.

3. The method of claim 2, wherein said oncogene is selected from the group consisting of tumor suppressors, tumor associated genes, growth factors, growth-factor receptors, signal transducers, hormones, cell cycle regulators, nuclear factors, transcription factors and apoptic factors.

4. The method of claim 3, wherein said tumor suppressor is selected from the group consisting of Rb, p53, p16, p19, p21, p73, DCC, APC, NF-1, NF-2, PTEN, FHIT, C-CAM, E-cadherin, MEN-I, MEN-II, ZAC1, VHL, FCC, MCC , PMS1, PMS2, MLH-1, MSH-2, DPC4, BRCA1, BRCA2 and WT-1.

11. The method of claim 4, wherein said tumor suppressor product is p53.

15. The method of claim 1, wherein said adenovirus particle is replication-defective.

16. The method of claim 15, wherein the replication defect is a deletion in the E1 region of the virus.

17. The method of claim 16, wherein the deletion maps to the E1B region of the virus.

18. The method of claim 17, wherein the deletion encompasses the entire E1B region of the virus.

19. The method of claim 18, wherein the deletion encompasses the entire E1 region of the virus.

20. The method of claim 1, wherein said promoter is selected from the group consisting of CMV IE, human or murine dectin-1, human or murine dectin-2, human CD11c, mammalian F4/80 and human or murine MHC class II.

21. The method of claim 20, wherein said promoter is CMV IE.

22. The method of claim 1, wherein said expression vector further comprises a polyadenylation signal.

24. The method of claim 1, wherein said cancer is selected from the group consisting of lung, head, neck, breast, pancreatic, prostate, renal, bone, testicular, cervical, gastrointestinal, lymphoma, brain, colon, skin and bladder.

26. The method of claim 1, wherein said expression construct is administered via injection.

27. The method of claim 26, further comprising multiple injections.

28. The method of claim 26, wherein the injection is performed local to a cancer, a pre-cancer or a tumor site.

29. The method of claim 26, wherein the injection is performed regional to a cancer, a pre-cancer or a tumor site.

30. The method of claim 26, wherein the injection is performed distal to a cancer, a pre-cancer or a tumor site.

31. The method of claim 1, wherein intradermal administration is via continuous infusion.

33. The method of claim 1, wherein said immune effector cells are CTLs.

34. The method of claim 1, further comprising administering to said subject at least a first cytokine.

35. The method of claim 34, further comprising administering to said subject a second cytokine, different from said first cytokine.

36. The method of claim 34, wherein said cytokine is selected from the group consisting of GM-CSF, IL-4, C-KIT, Steel factor, TGF-β, TNF-α and FLT3 ligand.

37. The method of claim 34, wherein said cytokine is administered as a gene encoded by said expression construct.

APPENDIX 2: PENDING CLAIMS

1. A method for treating a human subject having or suspected of having cancer or pre-cancerous disease comprising the steps of:

   (i) identifying a subject having or suspected of having cancer or pre-cancerous disease characterized by alteration or increased expression of a self gene product in at least some of the cancer or pre-cancerous cells in said subject; and

   (ii) intradermally administering to said subject an expression construct in an adenovirus particle comprising a self gene under the control of a promoter operable in eukaryotic dendritic cells, wherein the dendritic cells are infected by said construct,

whereby said self gene product is expressed by dendritic cells and presented to immune effector cells, thereby stimulating an anti-self gene product response.

2. The method of claim 1, wherein said self-gene product is an oncogene.

3. The method of claim 2, wherein said oncogene is selected from the group consisting of tumor suppressors, tumor associated genes, growth factors, growth-factor receptors, signal transducers, hormones, cell cycle regulators, nuclear factors, transcription factors and apoptic factors.

4. The method of claim 3, wherein said tumor suppressor is selected from the group consisting of Rb, p53, p16, p19, p21, p73, DCC, APC, NF-1, NF-2, PTEN, FHIT, C-CAM, E-cadherin, MEN-I, MEN-II, ZAC1, VHL, FCC, MCC , PMS1, PMS2, MLH-1, MSH-2, DPC4, BRCA1, BRCA2 and WT-1.

11. The method of claim 4, wherein said tumor suppressor product is p53.

15. The method of claim 1, wherein said adenovirus particle is replication-defective.

16. The method of claim 15, wherein the replication defect is a deletion in the E1 region of the virus.

17. The method of claim 16, wherein the deletion maps to the E1B region of the virus.

18. The method of claim 17, wherein the deletion encompasses the entire E1B region of the virus.

19. The method of claim 18, wherein the deletion encompasses the entire E1 region of the virus.

20. The method of claim 1, wherein said promoter is selected from the group consisting of CMV IE, human or murine dectin-1, human or murine dectin-2, human CD11c, mammalian F4/80 and human or murine MHC class II.

21. The method of claim 20, wherein said promoter is CMV IE.

22. The method of claim 1, wherein said expression vector further comprises a polyadenylation signal.

24. The method of claim 1, wherein said cancer is selected from the group consisting of lung, head, neck, breast, pancreatic, prostate, renal, bone, testicular, cervical, gastrointestinal, lymphoma, brain, colon, skin and bladder.

26. The method of claim 1, wherein said expression construct is administered via injection.

27. The method of claim 26, further comprising multiple injections.

28. The method of claim 26, wherein the injection is performed local to a cancer, a pre-cancer or a tumor site.

29. The method of claim 26, wherein the injection is performed regional to a cancer, a pre-cancer or a tumor site.

30. The method of claim 26, wherein the injection is performed distal to a cancer, a pre-cancer or a tumor site.

31. The method of claim 1, wherein intradermal administration is via continuous infusion.

33. The method of claim 1, wherein said immune effector cells are CTLs.

34. The method of claim 1, further comprising administering to said subject at least a first cytokine.

35. The method of claim 34, further comprising administering to said subject a second cytokine, different from said first cytokine.

36. The method of claim 34, wherein said cytokine is selected from the group consisting of GM-CSF, IL-4, C-KIT, Steel factor, TGF-β, TNF-α and FLT3 ligand.

37. The method of claim 34, wherein said cytokine is administered as a gene encoded by said expression construct.

[CANCER RESEARCH 63, 2145–2149, May 1, 2003]

Intradermal Injection, as Opposed to Subcutaneous Injection, Enhances Immunogenicity and Suppresses Tumorigenicity of Tumor Cells[1]

Bernard Bonnotte, Michael Gough, Vy Phan, Atique Ahmed, Heung Chong, François Martin, and Richard G. Vile[2]

Molecular Medicine Program, Guggenheim 18, Mayo Clinic, Rochester, Minnesota 55905 [B. B., M. G., V. P., A. A., H. C., R. G. V.], and INSERM U517, Faculty of Medicine, 21079 Dijon Cedex, France [B. B., F. M.]

ABSTRACT

Tumor cell immunogenicity depends heavily upon the microenvironment in which the cells grow. We have compared the tumorigenicity and immunogenicity of the same tumor cells when injected either into the dermis, a tissue containing numerous dendritic cells (DCs), or s.c., at a site which contains only few DCs. After s.c. injection, progressive tumors were constantly obtained, whereas most intradermal injections did not give rise to tumor and immunized animals against additional challenge. We present evidence that the high density of DCs at dermal sites facilitates the capture of tumor antigens and that local inflammation induces maturation of the DCs and their migration into draining lymph nodes.

INTRODUCTION

Several studies have demonstrated the trafficking of DCs[3] from the site of antigen capture to the draining lymphoid organs (1–3). Immature DCs such as Langherans cells capture tumor antigens and, under the influence of inflammatory stimuli, subsequently migrate to T-cell-rich areas such as lymph nodes where they can present antigen to naïve T cells and generate a tumor-specific immune response (2). The density of infiltrating DCs, the amount and form (4) of tumor antigen release, and the activation of these DCs by danger signals seem to be the most critical features for inducing an antitumor immune response (2). Using the experimental system PRO/REG (5), we previously demonstrated that tumor antigen release after s.c. injection is an important feature to induce an antitumor immune response (6).

In this study, we compared the tumorigenicity and immunogenicity of tumor cells injected either into the dermis, a tissue containing numerous DCs, or s.c., at a site which contains only few DCs. Most experimental rodent tumor models use s.c. injection of tumor cells to yield tumors in rodents. s.c. tissue is not rich in DCs, and s.c. injection does not induce local inflammation. In contrast to s.c. tissue, the skin is very rich in DCs such as Langerhans cells, which infiltrate the squamous epithelial cells of the epidermis and dermal DCs (3, 7, 8). Intradermal (i.d.) injection of tumor cells is not routinely practiced in the rodents, mainly because the thinness of dermis makes rigorous i.d. injection difficult and uncertain. We used PRO tumor cells, which after s.c. injection in rats, give rise to PRO, metastatic, and lethal tumors. PRO cells induce also peritoneal carcinomatosis when given i.p., multiple pulmonary metastases when administrated i.v., and liver nodules when injected into the spleen or the portal vein. s.c. PRO tumors induce immune tolerance because they enhance tumorigenicity and progression of highly immunogenic clones from the same tumor, the REG clones, which normally yield tumors that regress spontaneously in naïve rats (9).

In the present work, we demonstrated that i.d. administration of PRO tumor cells did not yield tumor in most injected animals. This tumor rejection is mediated by T cells as i.d. PRO tumor cells injected in nude rats always gave PRO tumors. Furthermore, all of the immunocompetent rats that have rejected the tumor cells after i.d. injection were vaccinated against additional s.c. tumor cell challenge. Immunohistological analyses performed on tumor injection sites showed a denser infiltration of DCs after i.d. tumor cell injection compared with s.c. injection. Furthermore, after i.d. but not s.c. injection of FITC-labeled tumor cells, DCs that have engulfed fluorescent tumor cell fragments could be recovered from the draining lymph nodes. Analysis of thymidine radioactive incorporation and IFN-γ production showed the presence of activated T cells in draining lymph nodes after i.d. but not after s.c. tumor cell injection. We also found that i.d. but not s.c. tumor cell injection induced local transcription of TNF-α, a major cytokine of inflammation that induces maturation of immature DCs into antigen-presenting cells. Thus, the high density of DCs and the local inflammation induced by the i.d. injection facilitate the capture of tumor antigen by immature DCs and their maturation into mature DCs and could be responsible for the subsequent rejection of a tumor cell line. These data show that the route of vaccine administration, as well as the site of the tumor growth, can have profound effects on outcome and may explain some of the variability that is often observed in *in vivo* vaccine experiments.

MATERIALS AND METHODS

Animals/Cell Lines/Tumorigenicity Assays. Nude rats and BD-IX rats were purchased from Charles River's Company (Wilmington, MA). In all of the animal experiments, we have observed the "Interdisciplinary Principles and Guidelines for the Use of Animals in Research, Marketing and Education" issued by the New York Academy of Sciences. The progressive variant cell line (PRO), established from a rat colon adenocarcinoma, was cultured as described previously (5). For the tumorigenicity assays, 1×10^6 PRO tumor cells in 100 μl of serum-free medium were injected s.c. or i.d. into the thoracic wall or i.d. in the ear. Rats were re-challenged by s.c. injection of 1×10^6 PRO tumor cells in the flank. Tumor volume was evaluated weekly, using a caliper to measure two diameters.

Histological Study of the Tumor Cell Injection Site. Animals were sacrificed 4 or 7 days after tumor cell injection. An immunohistochemical study was performed as described previously (1). Mouse mAb to rat immature DCs and monocytes (ED1), mature macrophages (ED2), MHC class II (OX-17), CD3 (R7/3), CD4 (W3/25) and CD8 (OX8), and IgG isotype control were obtained from Serotec (Oxford, United Kingdom).

Fluorescent Labeling of Tumor Cells. As previously described (1), 10×10^6 PRO cells were incubated for 30 min at 37°C with FITC (Fluka, Buchs, Switzerland) at a concentration of 500 μg/ml and washed until no fluorescence was detected in the supernatant.

Isolation of Lymph Nodes Cells. Rats were killed 4 days after i.d. or s.c. tumor cell injections. Draining lymph nodes were taken out, pooled for each experimental group, and mechanically disaggregated through a steel wire mesh. After washing, lymph nodes cells were suspended in RPMI 1640 supplemented with 1% sodium pyruvate, 50 μM 2-mercaptoethanol, and 10% FCS (Life Technologies, Inc., Rockville, MD).

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[3] The abbreviations used are: DC, dendritic cell; PRO, progressive; REG, regress; TNF-α, tumor necrosis factor α; mAb, monoclonal antibody; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Ags, antigens.

Proliferation Assay and IFN-γ Measurement. T cells recovered from the draining lymph nodes 4 days after i.d. or s.c. PRO tumor cell injection were suspended (10^6 cells/ml) in a round-bottomed 96-well plate (Nunclon, Roskilde, Denmark). They were cocultured in triplicate with mitomycin-preincubated PRO tumor cells (1×10^5 cells/ml). After 3 days of coculture, T-cell proliferation was measured as previously described (1), and IFN-γ concentrations were measured in the supernatant using ELISA kit for rat-IFN-γ (Quantikine; R&D, Oxon, United Kingdom).

Cytofluorimetry Analysis. Cells were washed in PBS supplemented with 0.5% BSA and 0.01% sodium azide, adjusted to 1×10^5 cells/100 μl, and analyzed with a FACScan. The CellQuest software was used to determine the percentage of fluorescent cells after the engulfment of FITC-labeled proteins and the number of MHC class II-positive cells.

Reverse Transcription-PCR Analysis. A piece of cutaneous tissue ($1 \times 1 \times 0.5$ cm) was excised 6, 24, and 48 h after i.d. or s.c. injection in 3 rats in each group. Total RNA was extracted (RNeasy Mini Kit; Qiagen, Inc., Valencia, CA) and pooled in each group. A total of 2.5 μg of total RNA was used in first strand cDNA synthesis, priming with oligo$(dT)_{15}$ (Boeringer Mannheim). PCR cycling conditions were 94°C for 10 min, 94°C for 1 min, 54°C for 45 s, and 72°C for 1 min, with steps 2–4 repeated 30 times. Primer sequences were as follows: *TNF-α*, 5′-GCACCATGAGCACGGAAAGC-3′ and 5′-GCTCACAGAGCAATGAC-3′; and *GAPDH*, 5′-CTGGTGCTGAGTATGTCGTG-3′ and 5′-CAGTCTTCTGAGTGGCAGTG-3′. Semiquantitative PCR amplifications were performed with coamplifying GAPDH (294 pb) and TNF-α (750 pb). PCR products were resolved on 1% agarose gel and stained with ethidium bromide.

RESULTS

Different DC Density in i.d. and s.c. Tissues. One million PRO tumor cells were injected s.c. or i.d. in naive syngeneic hosts. Only i.d. injection induced a swelling of the dermis and was followed by a moderate local inflammation during the first 2 days. To validate our observation and methodology, histological analyses were also performed just after i.d. or s.c. injection of tumor cells and were used to demonstrate the skin level (epidermidis, dermis, or hypodermis) in which the tumor cells were actually deposited. i.d. tumor cells (*) injection leads to a swelling of the dermis (Fig. 1*A*), whereas s.c. injections introduce tumor cells (*) under the hypodermis (Fig. 1*B*). Immunohistological analysis confirmed that 48 h after i.d. injection, the tumor cells were densely infiltrated by numerous ED-1-positive immature DCs (Fig. 1*C*), which are known to be located at the external dermis and the epidermis (3, 7, 8). In contrast, there were only a few DCs infiltrating the tumor cells after s.c. injection (Fig. 1*D*).

Tumor Cell Rejection after i.d. Injection. After s.c. injection, PRO tumor cells invariably yielded a tumor that continued to grow progressively. On the other hand, 11 of 18 rats (61%) that received PRO cells i.d. in the thoracic wall and all of the rats (15 of 15) that received PRO cells i.d. in the ear developed tumors that completely regressed in 2 or 3 weeks (Fig. 2*A*). Four weeks after i.d. or s.c. tumor cell injection in the thoracic wall, all of the rats were re-challenged against the same tumor cell line by a s.c. PRO tumor cell injection into the flank. All of the rats that rejected the tumor cells after i.d. injection were immunized, and no tumor was palpable at the site of the secondary challenge up to 6 weeks after the injection. In contrast, re-challenge produced PRO tumors in the flanks of all of the rats that developed PRO tumors after either s.c. (15 of 15) or i.d. (7 of 18) tumor cell injection at the thoracic site (data not shown). To demonstrate that this tumor rejection was mediated by the immune system, we injected PRO tumor cells i.d. in the thoracic wall (10 rats) or in the ear (5 rats) of nude rats. In all these nude rats tumor grew (Fig. 2*B*).

i.d. Injection of Tumor Cells Induces a Denser Infiltration of DCs at the Injection Site than s.c. Injection. To analyze the mechanisms of regression after i.d. injection, immunohistological analyses were performed on tumors on days 4 and 7 after i.d. or s.c. tumor cell injection into naïve rats (Fig. 3). At these times, tumor cells were easily identifiable according to their typical morphology and formation of poorly differentiated dense nodules. Tumors sites after s.c. injection (Fig. 3, s.c. column) were surrounded by a thin peritumoral halo containing a limited number of $ED1^+$ monocytes and immature DCs, sparse $ED2^+$ mature macrophages, nearly no $TCR^+/CD4^+$, and a few $TCR^+/CD8^+$ T cells, as well as some MHC class II^+ cells. In contrast, at day 4 after i.d. injection (Fig. 3, i.d. column), tumor nodules were densely infiltrated by $ED1^+$ and MHC-II^+ cells, which are a characteristic of rat DCs. At day 7, $ED1^+$ cells were replaced by a dense infiltration of $ED2^+$ mature macrophages within the tumor nodule. These macrophages were also $CD8^+$, a characteristic of activated rat macrophages (10). These $CD8^+$ cells were not T lymphocytes as most of the $CD8^+$ cells were not labeled with anti-TCR. Nevertheless, $CD8^+$ T cells were more abundant in the tumor site after i.d. injection.

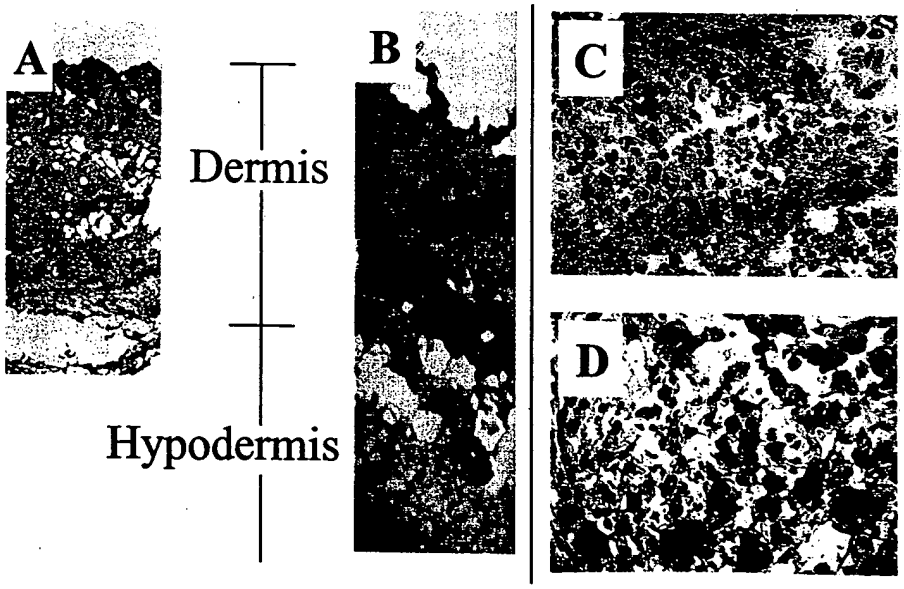

Fig. 1. Immunohistological analysis of cutaneous tissue. Histological and immunohistochemical analyses were performed on serial sections of rat skin just after i.d. (*A*, ×40) or s.c. (*B*, ×40) tumor cell injection using H&E labeling and 48 h after i.d. (*C*, ×250) or s.c. (*D*, ×250) tumor cell injection using ED-1 mAb that labeled immature DCs and monocytes.

i.d. Injection of FITC-labeled Tumor Cells Induces Migration of DCs, which Have Engulfed Fluorescent Tumor Cell Fragments, into the Draining Lymph Nodes. To follow the trafficking of tumor cell proteins during the initiation of the immune response, we labeled tumor cells with FITC. We have previously confirmed that FITC could induce a staining of tumor proteins and that FITC-labeled tumor proteins of an immunogenic tumor cell line could be engulfed by the immature tumor-infiltrating DCs, which then migrated to the draining lymph nodes (1). In this study, 4 days after i.d. injection of FITC-labeled PRO cells, low numbers of brightly fluorescent cells with dendritic morphology could be detected in the draining lymph nodes only after i.d. injection but not after s.c. injection using UV illumination (Fig. 4*A*) or fluorescence-activated cell sorting analysis (Fig. 4*B*). When analysis was restricted to gated larger cells, 3% of the larger cells were found to be fluorescent after i.d. injection, whereas no fluorescent cells were observed in the large cells recovered from the draining lymph nodes collected after s.c. injection of FITC-labeled PRO cells (Fig. 4*B*). To better characterize the migrated FITC-labeled cells, tumor-draining lymph nodes were dissociated and analyzed by flow cytometry. Nearly all FITC-labeled cells, recovered from lymph node draining sites of i.d. injected FITC-labeled PRO cells, had a dendritic morphology and expressed MHC class II molecules (Fig. 4*B*). These data strongly suggested that FITC-labeled cells found in the draining lymph nodes after i.d. injection of FITC-labeled PRO cells were DCs containing tumor cell proteins. T cells from draining lymph nodes were activated against PRO tumor cell Ags measured using proliferation (Fig. 4*C1*) and IFN-γ synthesis (Fig. 4*C2*) assays, only after i.d. tumor cell injection. Because of the moderate local swelling induced after an i.d. injection, we looked for synthesis of TNF-α. A significant amount of TNF-α mRNA was detected in tissues harvested 6 and 24 h after i.d. but not after s.c. injection (Fig. 4*D*).

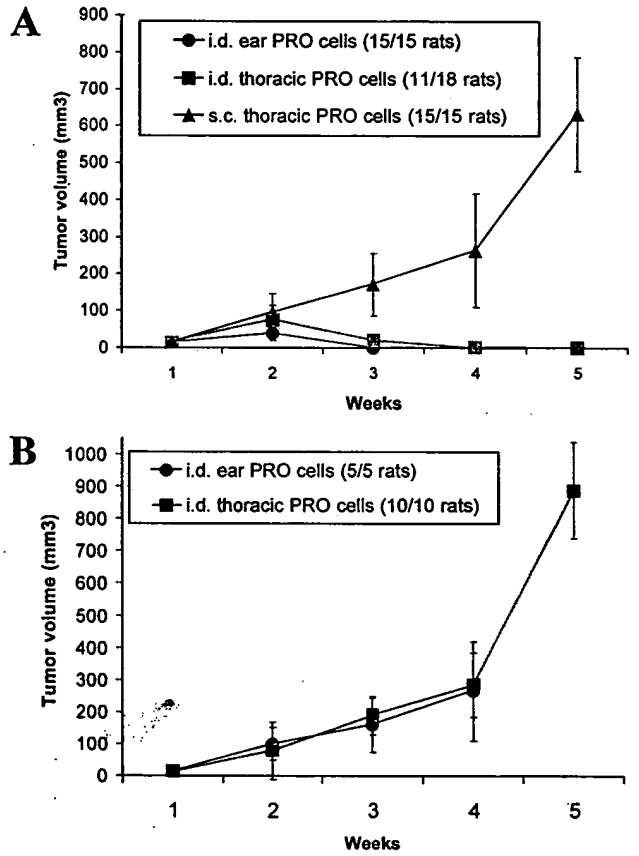

Fig. 2. Growth curves of the tumor after i.d. or s.c. tumor cell injection in naive rats. *A*, 10^6 PRO tumor cells were injected s.c. into the thoracic wall, i.d. in the thoracic wall, or i.d. in the ear of syngeneic BD-IX rats. All of the s.c. injections gave rise to progressive tumors, whereas all of the rats that had received i.d. PRO cells in the ear and 11 of 18 rats, which have received i.d. PRO cell into the thoracic wall, gave rise to tumors that completely regressed in 2–3 weeks. *B*, 10^6 PRO cells were injected i.d. in the thoracic wall or in the ear of nude rats. The curves represent the mean of two independent experiments. *Bars* indicate SD.

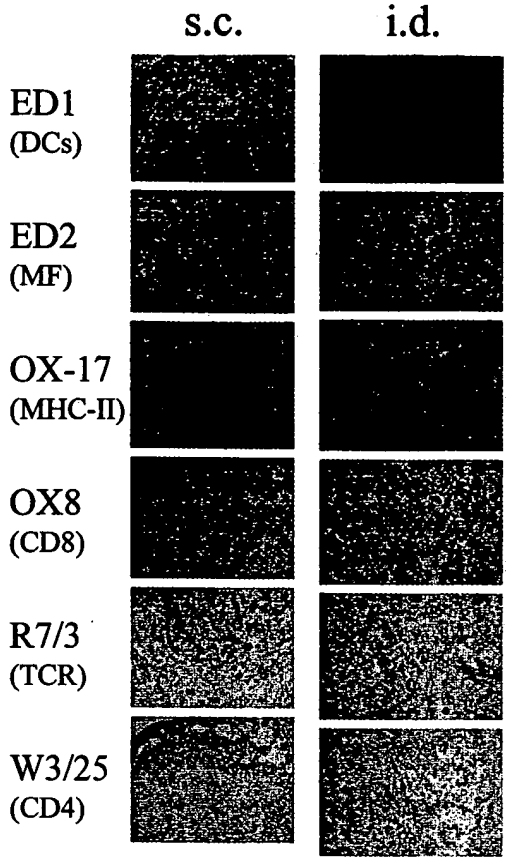

Fig. 3. Histological study of the tumor cell injection site. Animals were sacrificed 4 or 7 days after i.d. or s.c. tumor cell injection. Immunohistochemical analyses were performed on serial tumor sections, using mAbs that labeled monocytes and DCs (ED1), mature macrophages (ED2), MHC class II^+ cells (OX-17), $CD8^+$ T cells and macrophages (OX8), TCR^+ T cells (R7/3), and $CD4^+$ T cells (W3/25). Labeling of tumor slides after s.c. injection are in the column s.c., slides after i.d. injection are located in the column i.d. (×150). Similar results were observed in three pairs of tumor injection sites after i.d or s.c. injection.

DISCUSSION

In this study, we demonstrate that the site of cell injection significantly influences the tumorigenicity and immunogenicity of an experimental tumor model. When injected i.d., normally nonimmunogenic tumor cells can induce an immune response and be rejected. It has been previously reported that i.d. injection of various tumor cell lines, including sarcoma and leukemia, led in a variable proportion to a transient tumor followed by tumor regression and sometimes immunization against additional challenge with the same cell line through alternate routes such as i.p. or s.c. (11–14). However, the mechanism of this antitumor immune response induced by i.d. tumor cell injection has not been precisely defined. This rejection is immune-mediated because tumor cells were not rejected after i.d. injection by the rats devoid of a functional immune system such as nude rats. Skin is composed of the epidermis and the dermis both rich in DCs and the hypodermis, which is continuous with the s.c. tissue poor in antigen-presenting cells. The high concentration of DCs in the

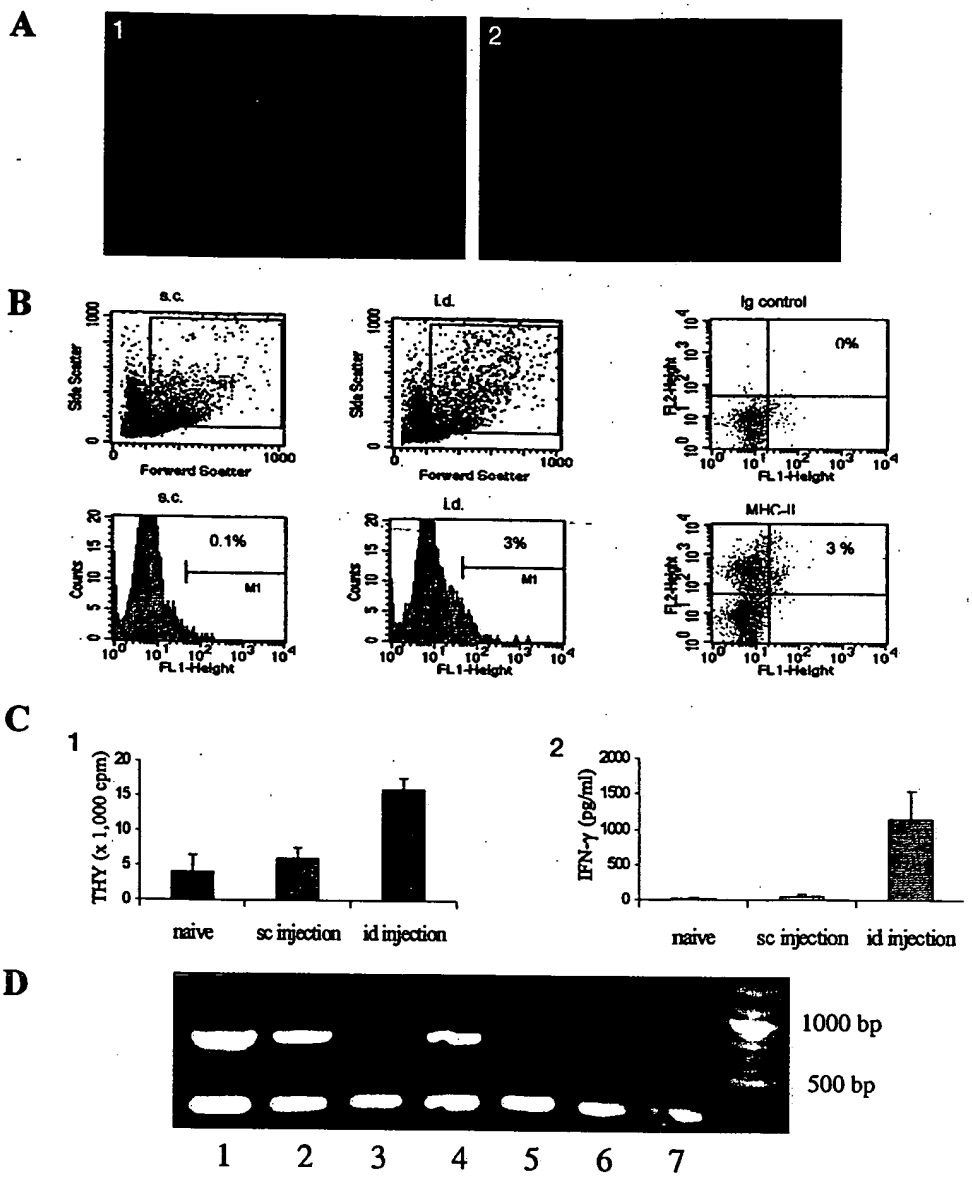

Fig. 4. Analyses of the DCs and the T cells recovered in the draining lymph nodes after i.d. or s.c. tumor cell injection. Four days after s.c. or i.d. injection of FITC-labeled PRO cells into the thoracic wall, the draining lymph nodes of 5 rats were pooled and disaggregated in a suspension. In very concentrated cell suspensions, a few cells were FITC positive in the suspension obtained from the draining lymph nodes only after i.d. injection (Fig. 4*A1*) and not in the lymph nodes cell suspension obtained after s.c. injection (Fig. 4*A2*). A cytometer analysis confirmed these results (Fig. 4*B*). An analysis of only the larger cells (gated) demonstrated that ~3% of these large cells were FITC positive (FL1-Height). Double labeling showed that nearly all of the fluorescent cells expressed MHC class II molecules (FL2-Height). T cells recovered from the draining lymph nodes 4 days after i.d. or s.c. PRO tumor cell injection were cocultured in triplicate with mitomycin-C-treated PRO tumor cells (1×10^5 cells/ml). We compared thymidine incorporation into T cells from draining lymph nodes after i.d. or s.c. tumor cell injection or without injection 3 days after the beginning of the mixed culture (Fig. 4*C1*). After 3 days of coculture, supernatants were collected and IFN-α concentrations was measured using ELISA kit (Fig. 4*C2*). Reverse transcription-PCR was performed with RNA isolated from tumor cell injection site, 6, 24, and 48 h after i.d. or s.c. injection. A semiquantitative assay was performed by coamplifying a fragment of GAPDH and TNF-α (Fig. 4D). *Lane 1:* RNA from activated macrophages as positive control; *Lanes 2, 4,* and *6:* RNA isolated, respectively, 6, 24, and 48 h after i.d injection; *Lanes 3, 5,* and *7:* RNA isolated 6, 24, and 48 h after s.c. injection.

dermis may facilitate capture of tumor antigens when nonimmunogenic tumor cells are i.d. injected (7–8). In contrast, an immunogenic tumor has been reported to grow when grafted to an extra lymphatic site. The loss of immunogenicity is believed to be attributable to the inability of tumor-antigen loaded DCs to reach the lymph nodes (15). Taken together, these data support the hypothesis that the immunogenicity of injected tumor cells depends on the numbers of DCs at the injection site. A slight modification of the depth of injection could influence the tumorigenicity and immunogenicity, and this fact, associated with the difficulty of i.d. injection in mice because of the thinness of the dermis, may explain the repeatedly observed phenomenon that only a certain proportion of mice of the same experimental group develop protection after tumor vaccination (11–14). This problem could be resolved by choosing an injection site where there is no s.c. tissue like the ear.

Increased tumor cell death induced by i.d. injection may explain the immunogenicity of this injection route with facilitating the capture of tumor Ags by DCs. Using a terminal deoxynucleotidyl transferase-mediated nick end labeling method, we did not find more apoptosis after i.d. than s.c. injection in the tumor site injection (data not shown).

To present antigens to naïve T lymphocytes, antigen-loaded DCs have to migrate into the draining lymph nodes. In this work, we used PRO cells, which had been previously labeled *ex vivo* with FITC (1), to visualize the migration of tumor cell proteins after i.d. injection of covalently FITC-labeled PRO cells. Four days after this injection, fluorescent DCs, identifiable by their morphology and MHC class II^+ phenotype, were found in the draining lymph nodes. By contrast, no fluorescent DCs were observed in the draining lymph nodes after s.c. injection of FITC-labeled PRO cells.

To present tumor antigens efficiently to T cells in the lymph nodes, immature DCs that infiltrate the tumor have to differentiate into mature DCs, which have reduced endocytosis capacity but overexpress MHC-peptide immunogenic complexes and costimulatory signals such as B7-1 and B7-2, on their plasma membrane. DC maturation can be induced by various inflammatory cytokines such as interleukin 1β and TNF-α (2). In addition, the proinflammatory cytokine TNF-α has been shown to be critical for inducing DC migration from the dermis to the lymph nodes (16, 17). Interestingly, only after i.d. but not after s.c. injection of PRO cells, local transcription of TNF-α mRNA was observed. Besides its role in the maturation of DCs and their migration into draining lymph nodes, TNF-α can

also attract immature DCs by inducing keratinocytes to secrete CCL20, the most potent known chemokine for attracting immature DCs (18). In the draining lymph nodes after i.d. tumor cell injection, DCs were potent tumor antigen-presenting cells as they induced specific T-cell proliferation. Activated T cells secrete IFN-γ, which could activate macrophages, the effector cell directly responsible for PRO tumor cell killing (19).

In conclusion, in contrast to s.c. PRO tumor cell injection, which gives rise to progressive and lethal tumors, i.d. injection of the same cell line induces an efficient antitumor immune response, leading to complete regression. Tumor regression correlated with a high density of DCs in the dermal tissue, which may facilitate the engulfment of tumor Ags. Furthermore, tumor cell i.d. injection induces synthesis of proinflammatory TNF-α, which may enhance DC maturation into fully efficient antigen-presenting cell and migration into the draining lymph nodes to activate tumor antigen-specific T cells. These data have important implications both for the interpretation of experimental results in which tumor cells are used and for the design of clinical trials using tumor vaccines. The richness of DCs in skin should make i.d. site a preferable site for injection of modified tumor cells in experimental and clinical trials using tumor vaccines.

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Adenovirus-mediated expression of melanoma antigen gp75 as immunotherapy for metastatic melanoma

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[illegible] Using this model [illegible] self-antigen that usually induces tolerance, and whether Ad vector-directed production of interleukin-2 (IL2) might augment this response. To evaluate this approach, Ad vectors were constructed containing the murine gp75 cDNA (Ad.gp75) and the human IL2 cDNA (Ad.IL2). Efficacy was evaluated in C57Bl/6 mice challenged i.v. with 10^5 B16 cells, using the number of lung metastases as the efficacy parameter. Naive control mice developed 175 ± 12 metastases by day 14. Controls receiving intranasal Ad.IL2 1 day after B16 cell injection, intraperitoneal (i.p.) mitomycin-C-treated B16 cells ± i.p. Ad.IL2 before B16 cell challenge and Ad.βgal-treated mice had similar numbers of metastases as controls [illegible]. [illegible] immunization with [illegible] reduction in the number of [illegible] metastases [illegible] of control). Addition of regional [illegible] lung) Ad.IL2 [illegible] Ad.gp75 preimmunization 1 day following tumor challenge provided further protection (18 ± 6, 10% of control). Depletion of CD4 and CD8 T-cell subsets effectively blocked the protective effect seen following immunization. Adoptive transfer of macrophage-depleted splenocytes from Ad.gp75-immunized mice similarly afforded significant protection against B16 tumor cell challenge. Further, serum obtained 21 days following Ad.gp75 immunization showed no detectable anti-gp75 antibody by immunoprecipitation. These results suggest that immunization with Ad.gp75 induces cellular immune responses that are capable of rejecting B16 melanoma in a host that is usually tolerant to gp75 antigen.

Keywords: *adenovirus; vaccine; melanoma; gp75; IL2; antigen*

Introduction

The immune response to human melanoma has been characterized in the past several years.[1,2] Specific antigens identified in murine and human melanomas include MAGE-1 and MAGE-3, MART 1/melan-A, tyrosinase, gp100, mutated CDK4, a 97 kDa cell surface glycoprotein melanotransferrin, a 66 kDa protein and a 75 kDa tyrosinase-related protein-1 (gp75).[3–14] Based on extensive data in murine models and in human cancers showing that the immune system can recognize antigens expressed by melanoma, therapeutic strategies have been designed to augment immune recognition of melanoma antigens utilizing attenuated tumor cells or cell lysates, either alone or mixed with immune adjuvants, tumor cells genetically modified to express cytokines, specific antigenic peptides, or gene transfer vaccination approaches using cDNAs coding for specific antigens.[15–47]

Gp75 is one of the melanoma antigens that has been evaluated in protein-based vaccine strategies.[41,48–51] The gp75 protein is an abundantly produced melanosome glycoprotein synthesized by pigmented melanocytes and melanomas but not in other cells.[48,52] The protein is an integral part of the melanosome membrane and has been identified as inducing antigen-specific B cell and T cell responses in individuals with melanoma.[51–53] Human gp75 has been purified and identified as the homologue of the mouse *b* (*brown*) locus gene product.[49,50,54] Similar to human gp75, murine gp75 is overexpressed in some murine melanomas.[50] The present study is directed toward the hypothesis that adenovirus (Ad) vector-mediated transfer of the murine gp75 cDNA can induce sufficient immunity in naive syngeneic C57Bl/6 mice to suppress the growth of B16 melanoma cells in lung following intravenous challenge with the tumor. Based on the knowledge that Ad-mediated *in vivo* transfer of transgenes often evokes both a cellular and a humoral immune response against the transgene,[55–60] we have constructed an Ad vector expressing the murine gp75 cDNA (Ad.gp75), and used this vector to immunize mice against syngeneic tumor expressing gp75. The data demonstrate this strategy is effective, appears to be dependent on cell-mediated immunity, and the antitumor immunity evoked by Ad.gp75 is further augmented by regional expression of interleukin-2 (IL2) delivered with an Ad vector.

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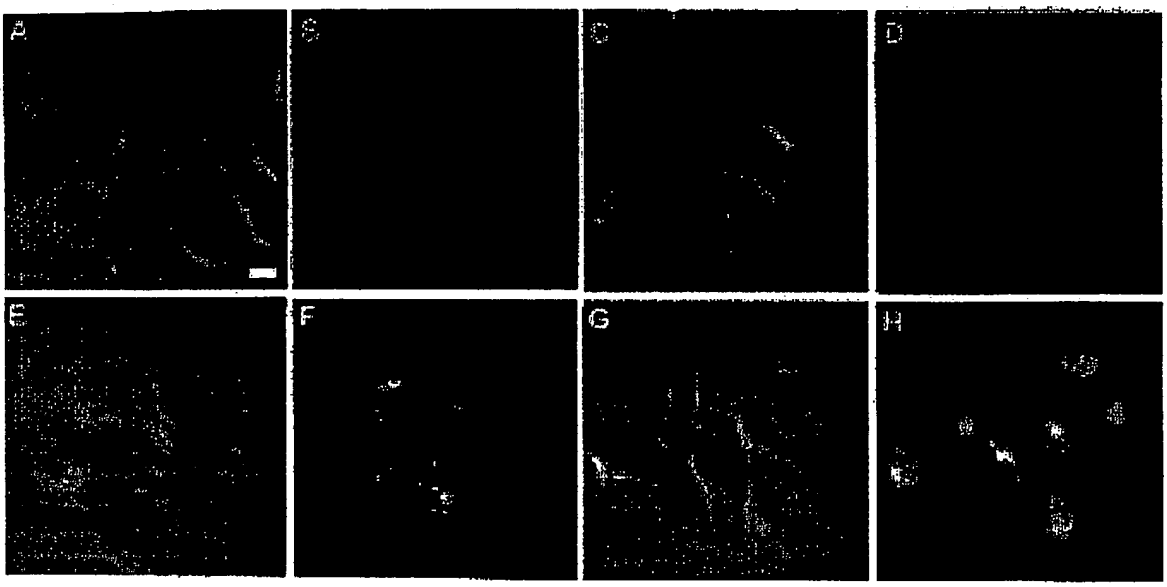

Figure 1 Expression of gp75 following in vitro infection with Ad.gp75. Human A549 lung adenocarcinoma cells were infected with Ad.gp75 (MOI 10); after 48 h the cells were evaluated for expression of gp75 using a gp75-specific monoclonal antibody and FITC-labeled second antibody. (A and B) Uninfected A549 cells. (C and D) A549 cells infected with control virus. (E and F) A549 cells infected with Ad.gp75. (G and H) B16 melanoma cells, which endogenously express the gp75 protein, as a positive control. (A, C, E and G) Differential interference contrast microscopy. (B, D, F and H) Immunofluorescence. Contrast enhancement to (B, D and F) are identical. The maximum fluorescence intensity in panel H was 2.6 times greater than (E) in the unmodified images. Bar = 10 μm.

Results

In vitro expression of gp75 protein

To evaluate the ability of Ad.gp75 to produce a gp75 protein in target cells, human A549 lung adenocarcinoma cells were infected with Ad.gp75 multiplicity of infection (MOI) 10 or control virus (MOI 10), with uninfected A549 cells serving as a negative control, and B16 murine melanoma cells known to express gp75 endogenously as a positive control. Immunostaining with an anti-gp75 antibody revealed a high level of Ad.gp75 protein in gp75-infected A549 cells, the expected expression of gp75 in the B16 cells, and no expression in cells infected with a control virus or uninfected cells (Figure 1). Fields from treated and control groups were imaged using both differential interference contrast microscopy and epifluorescence microscopy.

In vivo regional Ad-directed IL2 production

To evaluate the level of IL2 production in the lung following Ad.IL2 regional delivery, C57Bl/6 mice received a single transnasal dose of Ad.IL2 (10^9 p.f.u.; $n = 5$) or control virus (10^9 p.f.u.; $n = 3$) transnasally and IL2/mg lung protein was evaluated at 48 h, with naive mice serving as negative control. The Ad.IL2 vector yielded 3.8 ± 0.8 μg IL2/mg lung protein, whereas delivery of control virus yielded undetectable levels of IL2, similar to naive mice. To control for the theoretical variable of neutralizing immunity generated by the host in the setting of prior Ad.gp75 immunization, Ad.gp75 (10^9 p.f.u.) was delivered (by the intranasal route as in subsequent experiments) 7 days before Ad.IL2 administration. After 48 h, the IL2 levels in the lung were 4.3 ± 0.5 μg IL2/mg protein, similar to Ad.IL2 alone ($P > 0.1$) (Figure 2). There was insignificant systemic leak of IL2 in Ad.IL2-treated compared with naive mice measured by IL2 ELISA in the serum (0.2 ± 0.1 μg IL2/ml serum compared with control 0.1 ± 0.08 μg IL2/ml; $P > 0.1$). There was no

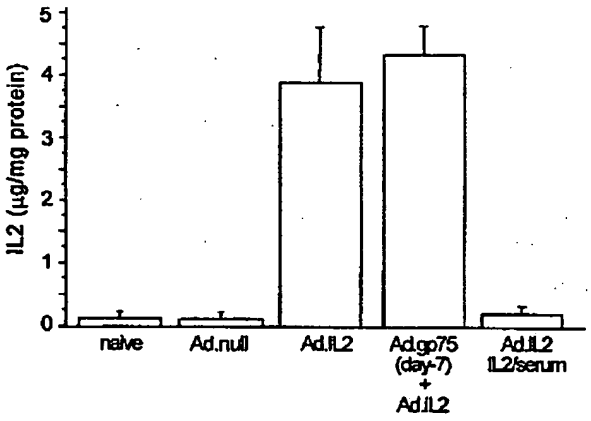

Figure 2 Ad-directed IL2 expression in vivo. Ad.IL2 or control virus AdCMV.βgal (both 10^9 p.f.u.) were delivered transnasally to the respiratory epithelium of C57Bl/6 mice; after 48 h, IL2 levels were quantified by ELISA in the lungs of mice. To control for the theoretical variable of reduced gene transfer secondary to neutralizing immunity following Ad.gp75 immunization, Ad.gp75 (10^9 p.f.u.) was administered intradermally 7 days before administration of Ad.IL2 (day −7); after 48 h, IL2 levels were quantified and compared with naive controls. The data are expressed as μg IL2/mg protein in the various groups. The data represent mean ± standard error of the mean of three animals per group in two independent experiments.

toxicity associated with IL2 expression in the lungs determined by no increase in mortality over 2 weeks after vector administration.

Effect of Ad-mediated regional delivery of IL2 against pulmonary metastases

Mice receiving i.v. administration of B16 cells were treated 1 day and 5 days after injection with Ad.IL2 (10^9 p.f.u.) transnasally, and the tumor load was evaluated at 14 days as the number of pulmonary metastases on the surface of the lung. Quantification showed regional delivery and expression of IL2 provided no therapeutic advantage when compared with control ($P > 0.1$; Figure 3).

Protective effect of Ad.gp75 immunization ± Ad.IL2

The ability of the Ad.gp75 vector to induce immunity directed against B16 cells was evaluated in a similar fashion as for Ad.IL2. Naive control mice developed 175 ± 12 metastases by day 14. In mice previously receiving a live tumor vaccine with B16 murine melanoma cells treated with mitomycin C, there was no significant protection against subsequent i.v. tumor challenge (reflected as the number of pulmonary metastases; $P > 0.1$ compared with naive animals) (Figure 4). B16 cells treated with mitomycin C delivered intraperitoneally (i.p.) in concert with i.p. Ad.IL2 (10^9 p.f.u.) offered no significant protection ($P > 0.1$). Similarly, Ad.βgal had no protective effect ($P > 0.1$). In contrast, a single intradermal immunization with Ad.gp75 7 days before tumor cell challenge provided marked protection against tumor cell challenge, reflected as a decrease in the number of metastases to 29 ± 4% of the number seen in naive controls ($P < 0.01$). The addition of regional Ad-directed IL2 expression within the lung provided additional significant protection to Ad immunization alone (10 ± 4% of control; $P < 0.01$ compared with naive; $P < 0.01$ compared with Ad.gp75 alone). As a further measure of tumor burden in Ad.gp75-immunized mice, comparisons were made with the number of metastases seen following dose escalating B16 tumor cell challenge. These mice showed a roughly linear increase in the number of metastases following B16 cell challenge between 10^4 and 10^5 cells, thus as expected the tumor burden in mice following Ad.gp75 immunization was significantly less than 5×10^5 cells ($P < 0.05$) and similar to a tumor cell challenge of 2.5×10^4 B16 cells ($P > 0.1$), and in mice treated with a combination of Ad.gp75 and Ad.IL2 was similar to 10^4 to 2.5×10^4 B16 cells ($P > 0.1$ both comparisons; not shown).

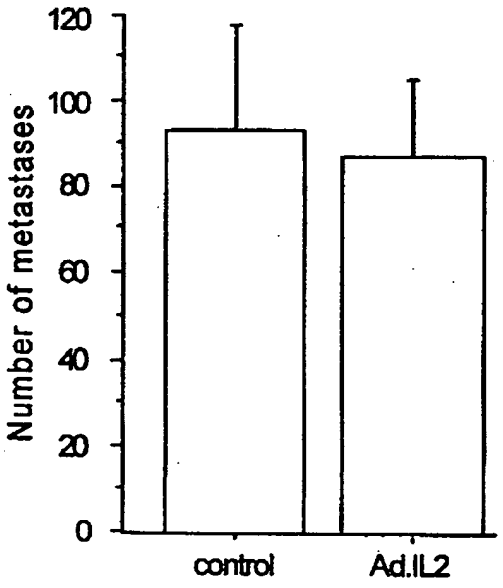

Figure 3 Effect of regional delivery of Ad.IL2 on B16 tumor growth into the lungs. B16 tumor cells (10^5) were administered i.v. to C57Bl/6 mice and 24 h later, Ad.IL2 (10^9 p.f.u.) was administered to the respiratory epithelium. The data are expressed as mean ± standard error of the mean of the number of pulmonary metastases in treatment and control mice with five mice in each group.

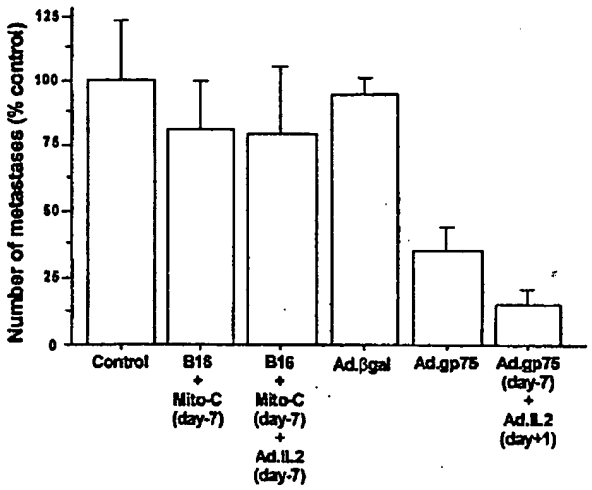

Figure 4 Protective immunity induced by Ad.gp75 alone and in combination with regional delivery of Ad.IL2. C57Bl/6 mice were immunized with Ad.gp75 (10^9 p.f.u., ID) 7 days before (day −7) i.v. challenge with syngeneic B16 murine melanoma cells (10^5 i.v.). In a subgroup of animals, Ad.IL2 (10^9 p.f.u.) was administered to the respiratory epithelium 1 day following tumor challenge (day +1). Experimental groups include naive control; i.p. immunization with mitomycin C-treated B16 cells 7 days before tumor challenge; i.p. immunization with mitomycin C-treated B16 cells + i.p. Ad.IL2 (10^9 p.f.u.) 7 days before tumor challenge; ID administration of Ad.βgal (10^9 p.f.u.); ID immunization with Ad.gp75 (10^9 p.f.u.) 7 days before tumor challenge; and mice immunized ID with Ad.gp75 (10^9 p.f.u.) 7 days before tumor challenge and treated with intranasal Ad.IL2 (10^9 p.f.u.) 1 day after tumor challenge. In all groups, the lungs were evaluated for the number of tumor nodules 14 days after tumor challenge. The data represent four separate experiments, each compared with its own naive control (minimum n = 8 animals per group), and are expressed as percentage of control.

Mechanisms of protective immunity

To explore the dependence of the tumor immunization on T cells, Ad.gp75-immunized mice were selectively depleted of either $CD4^+$ or $CD8^+$ T cells and assessed for their ability to reject B16 tumor cell challenge. Selective depletion was confirmed by cytofluorometric analysis on pooled residual splenocytes. The protective Ad.gp75 immunization was abolished, with a similar number of metastases developing in mice receiving anti-CD4 or anti-CD8 antibodies compared with control ($P > 0.1$; Figure 5). This is surprising, since only two-thirds of cells were shown to be depleted at the time of death. An isotype control had no significant effect on the protection

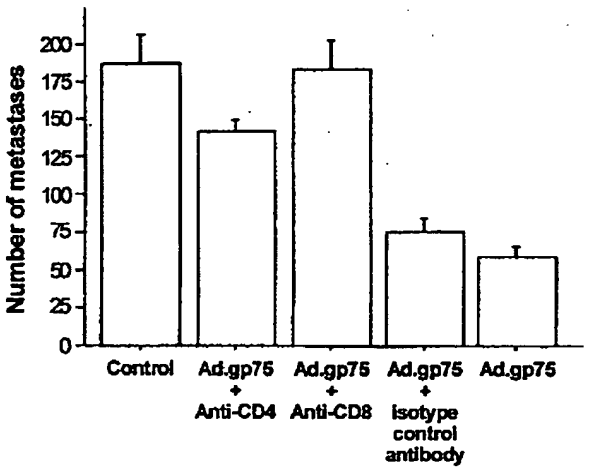

Figure 5 Selective depletion of CD4⁺ or CD8⁺ T cell subsets following immunization with Ad.gp75. Mice were immunized ID as described for Figure 4 and subgrouped to receive either anti-CD4, anti-CD8 monoclonal antibodies, isotype control monoclonal antibody, or no additional treatment. Mice were challenged with 10^5 B16 cells i.v. as above and the effect of T cell depletion was compared with naive control and Ad.gp75-immunized animals with intact cellular immunity.

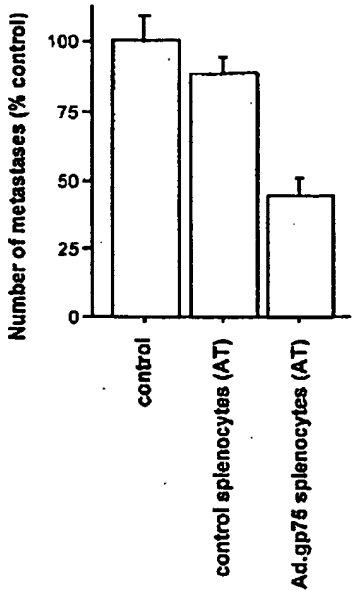

Figure 6 Adoptive transfer of splenocytes from Adgp75-immunized mice to naive mice before B16 tumor cell challenge. Mice were immunized ID as above and splenocytes harvested at day 7. Purified splenocytes were plated in vitro for 2 h to remove adherent macrophages and adoptively transferred by tail vein to naive mice at 10^7 cells per mouse. Twenty-four hours following mononuclear cell adoptive transfer, mice were challenged with 10^5 B16 cells i.v. The protection afforded by the transfer was measured as reduction in pulmonary metastasis at 14 days after tumor cell challenge and is expressed as percentage of control. AT, adoptive transfer.

afforded by Ad.gp75 immunization ($P > 0.1$ compared with Ad.gp75 immunization). To confirm the importance of cellular immunity in the suppression of B16 pulmonary metastases, splenocytes were harvested from mice immunized with Ad.gp75 7 days before or naive mice, depleted of macrophages, and 10^7 cells from either naive or immunized mice were adoptively transferred to naive mice which were then challenged with B16 tumor cells at 24 h. Consistent with cell-mediated mechanisms, mice receiving splenocytes from Ad.gp75-immunized mice developed only 43 ± 14% of metastases seen in naive controls (57% reduction; $P < 0.01$). Mice receiving control splenocytes showed a similar number of metastases to naive control ($P > 0.1$; Figure 6). Importantly, no evidence of humoral immunity against gp75 was detected in serum taken from mice 21 days after a single administration of Ad.gp75 using immunoprecipitation, determined by specific binding to S^{35}-methionine-labeled B16 lysates, whereas positive control TA99 antibody yielded the predicted 75 kDa band. This method has been shown sensitive enough to detect the presence of anti-gp75 auto-antibodies which correlates with antitumor effects seen in mice immunized with human gp75 protein or murine gp75 protein expressed in insect cells.[41] Consistent with these findings, immunization with syngeneic gp75 protein did not induce auto-antibodies to gp75.[41]

Discussion

The current study evaluates the potential of immunotherapy directed toward metastatic melanoma using adenovirus-mediated *in vivo* delivery of the cDNA encoding the wild-type murine gp75 melanoma-associated antigen to syngeneic mice. Using the number of pulmonary metastases as a measure of tumor burden, the data show a single intradermal injection of the Ad vector Ad.gp75 effectively protects mice against intravenous challenge of murine B16 melanoma cells which express gp75. Depletion of CD4⁺ and CD8⁺ lymphocytes abbrogated this antitumor effect suggesting an integral role for cell-mediated immunity. The suggested mechanism of cell-mediated immunity was further supported by the finding that adoptively transferred splenocytes from Ad.gp75-immunized mice to naive mice also afforded a high level of protection against subsequent tumor cell challenge. Consistent with these observations the antitumor effect was augmented regionally by the addition of Ad-mediated delivery of the human IL2 cDNA to the lungs of immunized mice. In contrast, Ad.IL2 offered no therapeutic advantage without prior Ad.gp75 immunization.

gp75 Antigen in Immunotherapy

The human and murine gp75 antigen (also referred to as the tyrosinase-related protein-1; TRP-1) are related 75 kDa glycoproteins found in mice on the murine *brown* locus.[41,48–51,54] Both human and murine gp75 are abundant in pigmented melanocytes and nevi, primary melanoma, and metastatic melanoma (melanosomal origin), and are not expressed in other human tissues. Gp75 was originally identified in association with high affinity IgG auto-antibodies in a patient with melanoma,[53,61] and has subsequently been shown to induce a specific T cell response which is HLA-A31 restricted.[51] The potential of

the gp75 antigen for immunotherapy is suggested by the finding that a modified form of the murine gp75 protein, as well as the human gp75 protein, possessing 80.2% homology to the murine gp75, is effective at inducing protective immunity against B16-derived pulmonary metastases.[41] Interestingly, the unmodified wild-type murine gp75 protein has not been shown to induce protective immunity.[41] Other than gp75, a variety of melanoma antigens have been identified including MAGE-1 and MAGE-3, MART 1/Melan-A, tyrosinase, gp100 and mutated CDK4, and are currently being evaluated in peptide-based, or vector-delivered gene-based vaccination trials.[39,47]

Ad-mediated transfer of the gp75 cDNA

Adenovirus-mediated gene-based vaccines take advantage of the high efficiency of gene transfer *in vivo* of this vector and capitalize on the host cellular immune responses known to be directed against Ad vector transgenes.[55–60,62,63] This paradigm has been evaluated using vectors carrying genes for a variety of foreign proteins; in these studies *in vivo* delivery of recombinant Ad vectors generates immunity against xenogenized tumor cells expressing the foreign antigen leading to rejection of the modified tumor cells.[62] Ad-mediated delivery of the human melanoma antigen gp100 cDNA can effectively immunize mice against murine tumors expressing murine gp100, which has a high degree of homology with the human gp100.[46] The current study extends this paradigm by showing Ad-mediated *in vivo* delivery of an unmodified autologous cDNA is capable of inducing protective immunity breaking tolerance against a tumor expressing the autologous antigen. The finding that Ad.gp75, carrying the unmodified wild-type murine gp75 cDNA, is capable of effectively vaccinating syngeneic mice against challenge of B16 melanoma expressing the autologous gp75 is in contrast to the finding that wild-type gp75 peptide vaccination does not appear to be effective.[41] The reasons the Ad vector gene-based gp75 vaccine is capable of inducing protective immunity to an autologous antigen remain to be evaluated, but differences from peptide-based vaccines may result from the protracted expression of antigen following gene delivery in the setting of an inflammatory response known to result from Ad vector infection.[57,64] The kinetics of gene expression, optimal dosing and route of administration of the vector, the potential for repeated administration, as well as comparative information on gene delivery of various isoforms of the gp75 antigen (modified murine *versus* human gp75) remain to be evaluated.

Augmentation of Ad.gp75 immunization with regional delivery of Ad.IL2

Although single administration of Ad.gp75 was capable of protecting against a subsequent tumor burden by 68% compared with control, some tumor burden remains in these animals. In the context that cellular mechanisms appear to be invoked (as demonstrated by the response to depletion of $CD4^+$ or $CD8^+$ lymphocytes), the addition of IL2 is intended to supplement the stimulatory signals to effector cells, thus leading to further activation and clonal expansion of antitumor cellular immune processes.[65,66] To approach this concept, we attempted to avoid the toxicity associated with systemically administered IL2,[67,68] by delivering IL2 directly to the target organ, thus limiting systemic side-effects. The vector Ad.IL2 produced high levels of IL2 in the area to which the vector was directed, but showed no significant systemic leak of IL2 and animals all appeared healthy throughout the 2 week treatment period. Although some studies have shown *ex vivo* modification of tumor cells with the IL2 cDNA[26,69,70] or that *in vivo* viral vector-mediated production of IL2 by established tumor can inhibit tumor growth,[71–77] regional delivery of Ad.IL2 to the respiratory epithelium alone offered no significant therapeutic benefit against pulmonary metastasis to mice receiving an intravenous tumor burden. This therapy was, however, able to complement Ad-mediated gp75 gene expression, providing further significant protection against tumor challenge. In this context, Ad-mediated gene-based gp75 vaccines together with regional Ad vector-delivered IL2 may be a useful adjuvant to current therapy for limited disease for prevention of pulmonary metastasis.

Materials and methods

Adenovirus vectors

The $E1a^-$, partial $E1b^-$, partial $E3^-$ Ad vectors Ad.gp75 and Ad.IL2 were constructed using the Ad5 pJM17 backbone recombination plasmid pNY.102 (for Ad.gp75), and the recombination plasmid pCMV.S2+ for Ad.IL2.[78] The Ad.gp75 vector expression cassette (right to left orientation) contains the cytomegalovirus immediate-early (CMV) promoter, followed by an artificial splice sequence, the murine gp75 cDNA, and the SV40 polyadenylation sequence. The Ad.IL2 vector is identical except for the human interleukin 2 (IL2) cDNA (gift of Bernard Gansbacher, Memorial Sloan-Kettering Cancer Center, New York, NY, USA) in the expression cassette in a left to right orientation in place of the gp75 cDNA. Both vectors were plaque purified, amplified, purified and titrated as previously described.[79,80] The control vectors Ad.null, similar to therapeutic vectors but carrying no transgene, and Ad.βgal expressing the *E. coli* β-galactosidase (βgal) gene, were amplified as previously described.[78]

Ad.IL2 production of human IL2 in vivo

To test Ad.IL2 directed expression of human IL2, Ad.IL2 (10^9 p.f.u.; $n = 5$) or control virus Adβgal (10^9 p.f.u.; $n = 3$) were delivered transnasally to the respiratory epithelium of C57BL/6 mice. To control for the theoretical variable of reduced gene transfer secondary to neutralizing immunity following Ad.gp75 immunization that could potentially limit expression of Ad.IL2,[58] Ad.gp75 (10^9 p.f.u.) was delivered intradermally 7 days before IL2 delivery ($n = 3$). At 48 h, the lungs were removed, homogenized in protein lysis buffer (10 mM Tris-HCl, 2% Triton X-100, 0.025% sodium azide, 0.14 M NaCl, 1 mM phenylmethanesulfonyl fluoride, 30 μM leupeptin, 30 μM apoprotinin, 30 μM peptistatin) (Sigma, St Louis, MO, USA) using a Polytron homogenizer. Total protein was quantified using a BioRad protein assay (BioRad, Hercules, CA, USA), and IL2 tissue levels were quantified per mg protein using an enzyme-linked immuno assay (ELISA; R&D, Minneapolis, MN, USA) and compared with naive control. To assess whether there was significant systemic leak of IL2 following regional delivery of Ad.IL2 to the lungs, blood was taken from the above ani-

mals at the time of death by right heart puncture and the amount of IL2 per ml serum was assessed by ELISA as described above.

Immunostaining for Ad-mediated gp75 transfer in vitro

To evaluate the expression of gp75 antigen in transduced target cells, A549 human lung adenocarcinoma cells (ATCC, Rockville, MD, USA) were infected with Ad.gp75 (MOI 10). Negative controls include uninfected cells and A549 cells infected with Ad.βgal (MOI 10). B16 murine melanoma cells endogenously expressing gp75 served as a positive control. The cells (10^5) were infected in suspension and plated (on a chamber slide) for 48 h and fixed with 4% paraformaldehyde. The paraformaldehyde was inactivated with 5 mM NH_4CL, the cells permeabilized with 0.01% saponin with 1% bovine serum albumin in phosphate-buffered saline (pH 7.4), stained with the TA99 anti-gp75 monoclonal antibody (1:200 dilution), and counterstained with a fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse secondary antibody.[81] Representative fields were imaged with a Nikon Microphot SA, equipped with a ×40, 0.7 NA objective lens, Nikon FITC epifluorescence filter cube, and differential interference contrast optics (Nikon, Melville, NY, USA). Images were collected with a cooled charge-coupled device camera (Princeton Instruments, Trenton, NJ, USA).

Cell line and animal model

The B16 murine melanoma cell line is a weakly immunogenic tumor which expresses the gp75 protein in melanosomes within the cells and on the cell surface.[61] The cell line is syngeneic to the C57Bl/6 mouse and reliably produces pulmonary metastases following i.v. injection.[82] To establish metastases, 10^5 B16 cells were injected by the lateral tail vein to C57Bl/6 mice (Charles River, Wilmington, MA, USA). The numbers of metastases were quantified by counting the black tumor nodules on the surface of the lung.[82]

In vivo delivery of Ad.IL2 to the lungs of tumor bearing mice

Mice were challenged with B16 tumor (10^5 i.v.) followed 1 day later by Ad.IL2 to the respiratory epithelium (10^9 p.f.u. via nasal instillation; $n = 4$), or no therapy ($n = 5$), and repeated following a 5 day interval. Mice were killed 14 days following tumor cell delivery and the numbers of pulmonary metastases were counted under a dissecting microscope in both treatment and control (naive) groups.

Suppression of pulmonary metastasis using adenovirus-mediated immune modulation

Treatment groups include mice immunized intradermally (ID) 7 days before tumor challenge ('day −7') with Ad.gp75 (10^9 p.f.u.) with no additional therapy ($n = 9$), or Ad.gp75 plus regional administration of Ad.IL2 to the respiratory epithelium 1 day following tumor challenge (10^9 p.f.u. via nasal instillation; $n = 10$). Control groups included naive C57Bl/6 mice, mice immunized with B16 cells treated with mitomycin C (Sigma, St Louis, MO, USA; 5×10^5 cells i.p., day −7), mitomycin C-treated B16 cells followed by Ad.IL2 (10^9 p.f.u., i.p., day −7) or mice immunized with Adβgal (10^9 p.f.u., ID, day −7). Lungs were removed 14 days after tumor cell challenge and the number of tumor nodules were counted under a dissecting microscope. The data represent the combination of four independent experiments each with independent controls (minimum $n = 8$ animals per experiment). To quantify further the protective effect of this therapy, a titration of B16 tumor cells was performed to compare the relative tumor burden seen in Ad.gp75 immunized mice with an equivalent tumor cell challenge. Control naive mice received an i.v. injection of 10^5, 5×10^4, 2.5×10^4, and 10^4 B16 cells as described and the numbers of tumor nodules were evaluated at day 14.

In vivo depletion of T cell subsets

Ad.gp75-immunized mice were selectively depleted of $CD4^+$ or $CD8^+$ lymphocytes using monoclonal antibodies ($n = 7$ each group) and compared with immunized mice receiving no antibody ($n = 20$) and naive control ($n = 16$).[61] Mice received i.p. injections of 200 μg anti-CD4 (GK1.5, TIB-207 hybridoma, LT3T4; ATCC) anti-CD8 (TIB-105 hybridoma, Lyt-2; ATCC) or isotype control antibody (IgG_{2A} κ; PharMingen, San Diego, CA, USA) 2 days before and 2, 7 and 12 days after tumor cell challenge. Mice were evaluated for selective depletion of targeted lymphocyte populations by cytofluorometric analysis (Coulter, Miami, FL, USA). Residual spleen cells pooled from three mice from each group were stained with FITC-conjugated anti-mouse CD3a antibody, PE-conjugated anti-mouse CD8e or conjugated anti-mouse CD4 antibody, or a combination of anti-CD3/anti-CD4 or anti-CD3/anti-CD8 (Pharmingen). At the time of death there was a 65% reduction in the number of $CD4^+$ lymphocytes following treatment with anti-CD4 antibody compared with mice immunized with Ad.gp75, and a 66% reduction in the number of $CD8^+$ lymphocytes following treatment with anti-CD8 antibody compared with naive, and a 73 and 74% reduction compared with mice immunized with Ad.gp75, respectively.

Adoptive transfer of mononuclear splenocytes

Mice were immunized with Ad.gp75 as described above. At 7 days, mononuclear splenocytes from immunized and naive mice were isolated as previously described.[60] Five spleens from each group were harvested fresh and placed in a tissue culture dish with complete Dulbecco's modified Eagle's medium (DMEM; Biofluids, Rockville, MD, USA). The spleens were minced and ground, sheared with a 19-gauge needle, passed through a 200 μm mesh to remove fibrous tissue. Cells were pelleted then resuspended in DMEM plus 10% fetal bovine serum (Gibco BRL, Grand Island, NY, USA), and 50 units/ml penicillin G, 50 μg/ml streptomycin (Gibco BRL) ('complete media'; $5–8 \times 10^7$ cells per spleen). Live lymphocytes were separated from dead cells and RBCs using a Ficoll-Paque (Pharmacia, Piscataway, NJ, USA) density separation technique, washed, then resuspended in complete media. Pooled splenic mononuclear cells from each group were plated in tissue culture for 2 h to allow macrophages to adhere to the plate. Nonadherent cells from each group were removed and resuspended at 10^7 cells/200 μl PBS, then adoptively transferred to naive mice via tail vein injection. At 24 h mice were challenged with 10^5 B16 melanoma cells i.v. as previously described. Tumor burden was evaluated at 14 days by counting the number of pulmonary nodules as described above.

Detection of serum anti-gp75 antibodies

Mice were immunized with 10^9 p.f.u. Ad.gp75 ID and serum collected 21 days following immunization and tested by immunoprecipitation as previously described.[83] B16 cells (5×10^6) were preincubated in methionine-free medium (3 h, 37°C) then metabolically labeled with 300 μCi ^{35}S-methionine/10^7 cells (specific activity 1140 Ci/mmol; 7.9 mCi/ml; New England Nuclear, Boston, MA, USA; 18 h, 37°C). Cells were washed and solubilized at 10^7 cells/ml in lysis buffer (150 mM NaCl, 10 mM Tris-HCl, pH 7.6, 1% NP-40, 2 mM ethylenediaminetetraacetate (EDTA); 30 min, 4°C). Serum (5 μl) or TA99 (1 μl) MAb was incubated with 150 μl lysate at 4°C for 30 min. Immune complexes were recovered on 30 μl protein A bound agarose beads (Pharmacia, Piscataway, NJ, USA) washed (15 mM NaCl, 10 mM Tris-HCl, pH 7.6, 1% NP-40, 5 mM EDTA) and eluted with Laemmli buffer (0.01 M Tris-HCl pH 7.2, 2% sodium dodecyl sulfate (SDS), 12.0 mg/ml dithiotreol, 15% (w/v) sucrose, 0.01% pyronin Y; 5 min, 100°C). Samples were analyzed on a 10% SDS-polyacrylamide gel. Radioactive bands were revealed by fluorography.

Statistical analysis

All data are presented as mean ± standard error of the mean and all comparisons were made using the unpaired two-tailed Student's *t* test.

Acknowledgements

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